

The active site of methanol dehydrogenase contains a disulphide bridge between adjacent cysteine residues

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Adjacent cysteine residues can only form disulphide bridges in a distorted structure containing a *cis*-peptide link. Such bridges are extremely uncommon, identified so far in the acetyl choline receptor alone where the structure of the bridge is undetermined. Here we present the first molecular description of a disulphide bridge of this type in the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens*. We show that this structure occurs in close proximity to the pyrrolo-quinoline quinone prosthetic group and a calcium ion in the active site of the enzyme. This unusual disulphide bridge appears to play a role in the electron transfer reaction mediated by methanol dehydrogenase.

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Methanol oxidation in methylotrophic bacteria growing on methane or methanol is catalysed by a soluble NAD⁺-independent methanol dehydrogenase (MDH)^{1,2}. This is the first protein in a soluble electron transport chain in the periplasm of these bacteria. The electron acceptor for MDH is cytochrome *c*_L (ref. 3). MDH is the dehydrogenase in which the prosthetic group pyrrolo-quinoline quinone (PQQ) was first characterised and its structure determined⁴⁻⁶. Dehydrogenases containing this prosthetic group are called quinoproteins.

The quinoprotein MDH from *Methylobacterium extorquens* is an $\alpha_2\beta_2$ tetramer⁷, containing at least one calcium ion and two molecules of PQQ⁸ (Fig. 1). MDH has 4 cysteines in the α subunit (Cys 103,104,386,415) and 2 in the β subunit (Cys 6,12)^{7,9}, and preliminary results indicate that all the cysteines might be involved in formation of disulphide bonds (unpublished data). If this is the case then the adjacent cysteines, Cys 103 and Cys 104 are of particular interest. It has been predicted that ring closure by an S-S bridge between adjacent cysteine residues is only likely to occur in a distorted structure when the peptide bond linking the cysteines is in a *cis* conformation^{10,11}. Although subsequently confirmed in model peptides^{12,13}, it has become clear that such disulphide bridges are extremely uncommon and have been identified in only a single native protein, the acetyl choline receptor, the structure of which has not been determined¹⁴. Here we present the first description of an active enzyme, methanol dehydrogenase, that contains a disulphide bridge linked by a *cis*-peptide bond.

Eight-membered ring

An interpretable electron density map of MDH from *M. extorquens* was calculated at 3 Å resolution using phases determined by multiple isomorphous replacement (MIR) and symmetry averaging¹⁵. This map shows that in the β -chain Cys 6 is linked to Cys 12 and in the α -chain Cys 386 is linked to Cys 415, in two conventional intrachain disulphide bridges. The electron density around the remaining α -chain cysteines, 103 and 104, is in the form of a small diameter ring (Fig. 2), which includes some strong density, characteristic of sulphur atoms. The clear interpretation of this feature is that the adjacent Cys 103 and Cys 104 residues are linked together in a disulphide bridge. We can be sure that neither Cys 103 nor Cys 104 are free thiols in the crystal because the thiol reagent ethyl mercuric phosphate, which was used to form one of the isomorphous derivatives, gained access to the active site but bound near the calcium ion (see below) and not to the cysteines.

Theoretical analysis of the conformation and energetics of a disulphide bridge formed between adjacent cysteine residues^{10,11} suggests that, as well as requiring a linking peptide in highly distorted *cis*-conformation, a non-planar ring may also be necessary. X-ray analysis of crystals of cyclic Cys-Cys dipeptides have confirmed the presence of a linking *cis*-peptide but not the non-planarity of the eight-membered ring^{12,13}. These analyses demonstrate the presence of two conformers that have different dihedral angles around the two sulphur atoms. The electron density in the proximity of residues 103 and

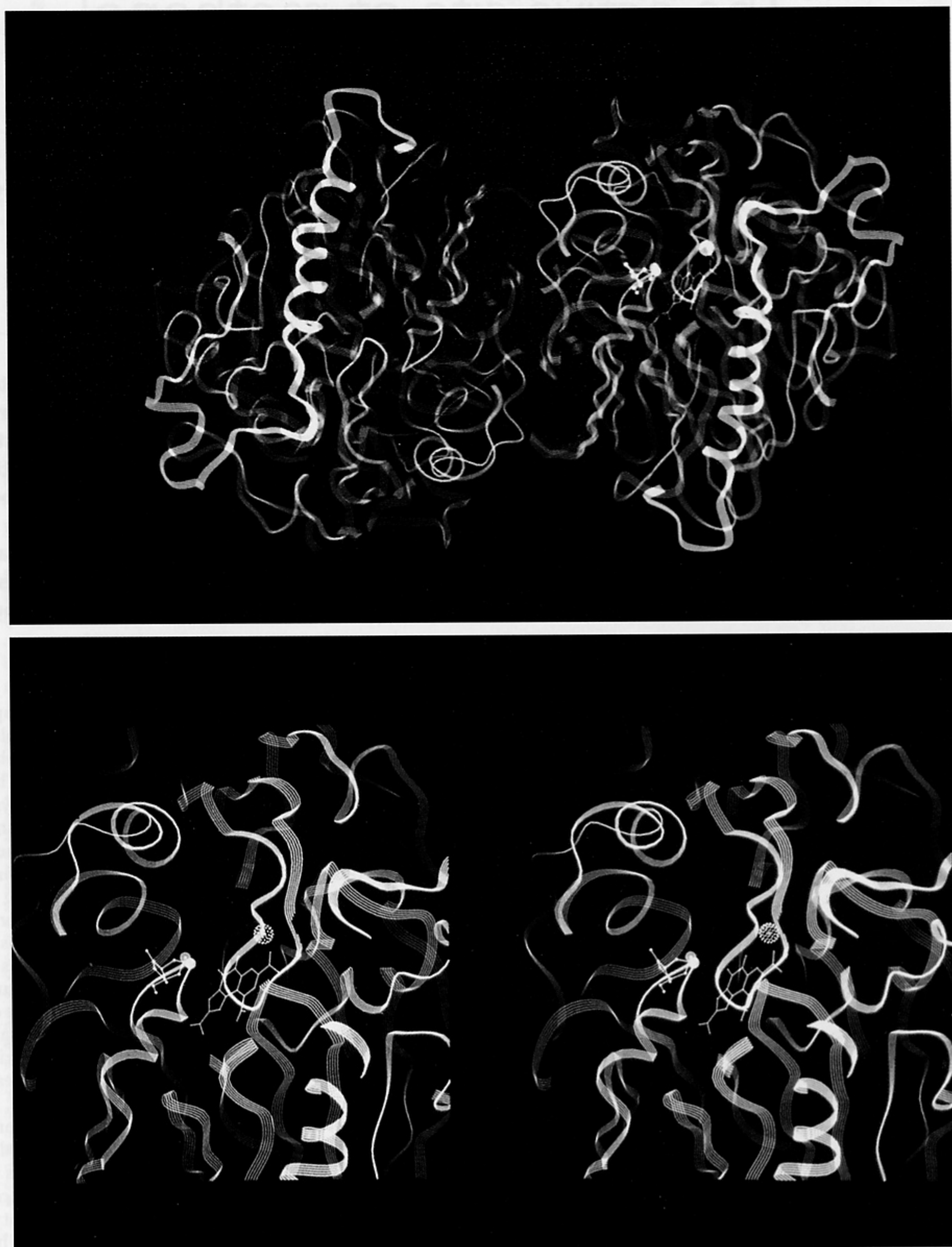


Fig. 1 a, The three-dimensional structure of the $\alpha_2\beta_2$ tetramer of MDH from *Methylobacterium extorquens* viewed down the molecular 2-fold axis. The two 599-residue α -chains are coloured pink and purple while the two non-globular 74-residue β -chains are both shown in yellow. Atom representations of PQQ are shown in orange, the Cys 103-Cys 104 residues in yellow and the calcium ion as the blue sphere in one α -chain. The overall fold of this MDH is similar to the MDH from *Methylophilus methylotrophus*²⁰. b, A close-up view, in stereo, of the active site region in the same orientation as (a).

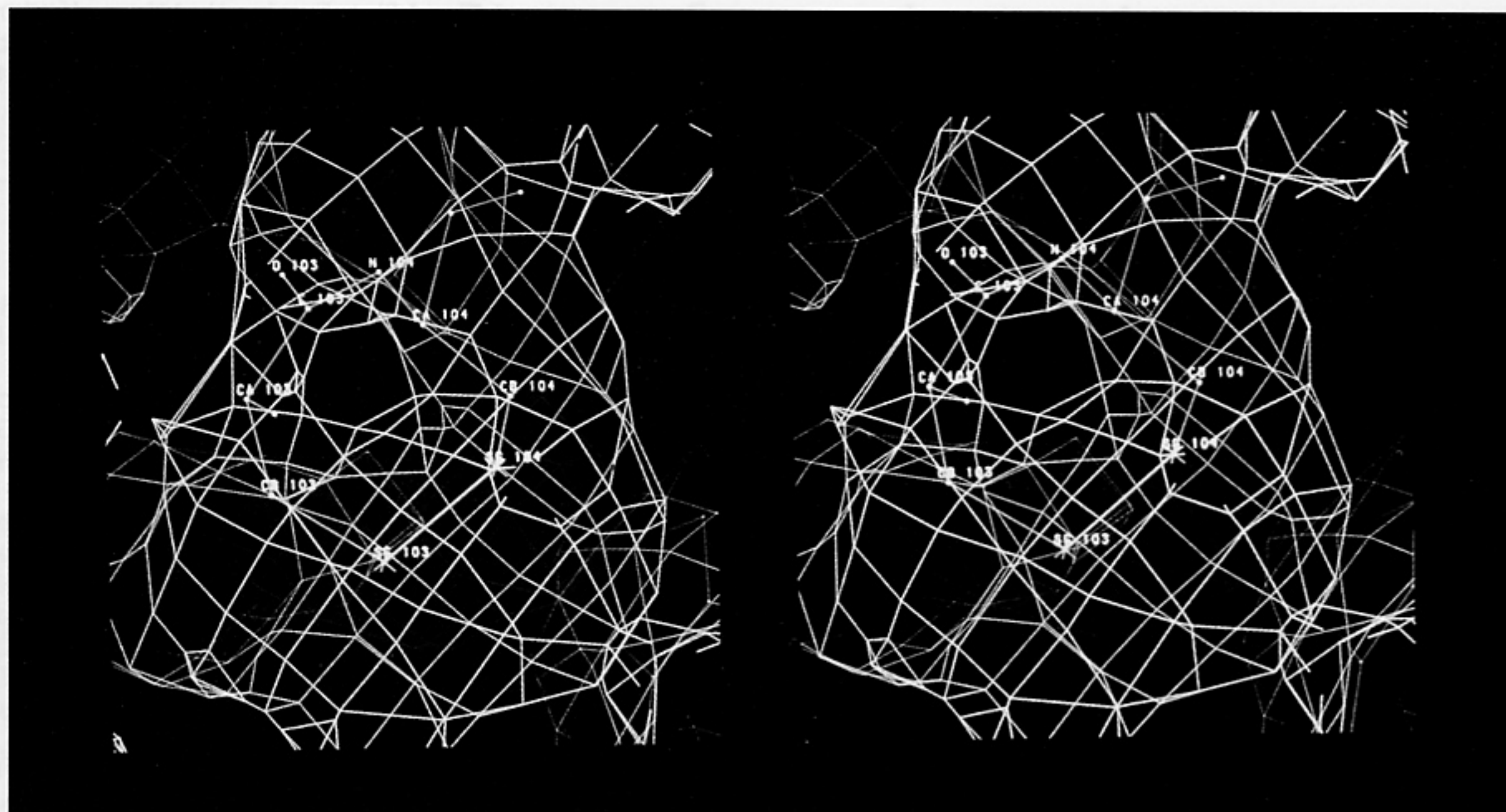


Fig. 2 A stereo view of the electron density around Cys 103 and Cys 104 contoured at 0.7σ (green) and at 3.5σ (red), where σ is the r.m.s. standard deviation in the electron density. The fit of the coordinates of the eight-membered ring of cyclo-L-cystine¹³ is shown in yellow.

104 can be fitted to an eight-membered ring with the same configuration described for cyclo-L-cystine¹³, thus confirming the *cis*-conformation for the linking peptide in the enzyme (Fig. 2).

The presence of the Cys 103–Cys 104 disulphide bridge is further confirmed by the observation that one of the disulphide bridges in the α -chain can be readily reduced by treatment with 5mM dithiothreitol (DTT) for 1 hour with no further reduction when treatment was prolonged for 16 hours. The cysteines involved were identified by reduction, carboxymethylation with ¹⁴C-iodoacetate, trypsin digestion, and separation and sequencing of the single radioactive peptide. Its sequence, corresponding to a sequence in the MDH α -chain starting with Ala 100, was Ala Val Ala Cys* Cys* Asp Leu Val (where Cys* represents carboxymethylated cysteine). A similarly readily-

reducible disulphide bond has been identified in the agonist binding site of the acetylcholine receptor¹⁴, the only other native protein in which this type of bond has been found.

Role in electron transfer

The Cys 103–Cys 104 disulphide bridge is located in the enzyme's active site in close proximity to the PQQ prosthetic group (Fig. 3). Because a disulphide bridge between adjacent cysteine residues cannot fulfil the normal structural role of disulphides, we investigated the possibility that it plays a role in catalysis. When the disulphide bond is reduced by DTT, the enzymatic activity of MDH — transferring electrons to cytochrome *c*₁ — dropped to zero (Fig. 4). There is no loss of activity when measured with phenazine ethosulphate as the electron acceptor. The conclusion that the Cys 103–Cys 104 bridge plays a role in the electron transfer mediated by MDH is consistent with the conservation of the two equivalent cysteine residues in other quinoproteins in which electrons are transferred from PQQ to C-type cytochromes, and the absence of these cysteines in glucose dehydrogenase, whose electron acceptor, ubiquinone, is located in the membrane.

MDH contains Ca²⁺ (refs 8,16). Spectroscopic studies of a mutant form of MDH that lacks Ca²⁺ and is unable to oxidize substrate have demonstrated that Ca²⁺ is essential for maintaining the PQQ prosthetic group in its correct configuration⁸ and suggest that Ca²⁺ may have a catalytic role. The electron density maps of MDH contain an isolated density feature in the active site that is too high to be a water molecule. The size and environment of this density suggest that it represents the calcium ion. The ion is co-ordinated by 4 protein ligands, Glu 177, Asn 261, Asp 303 and the main-chain carbonyl

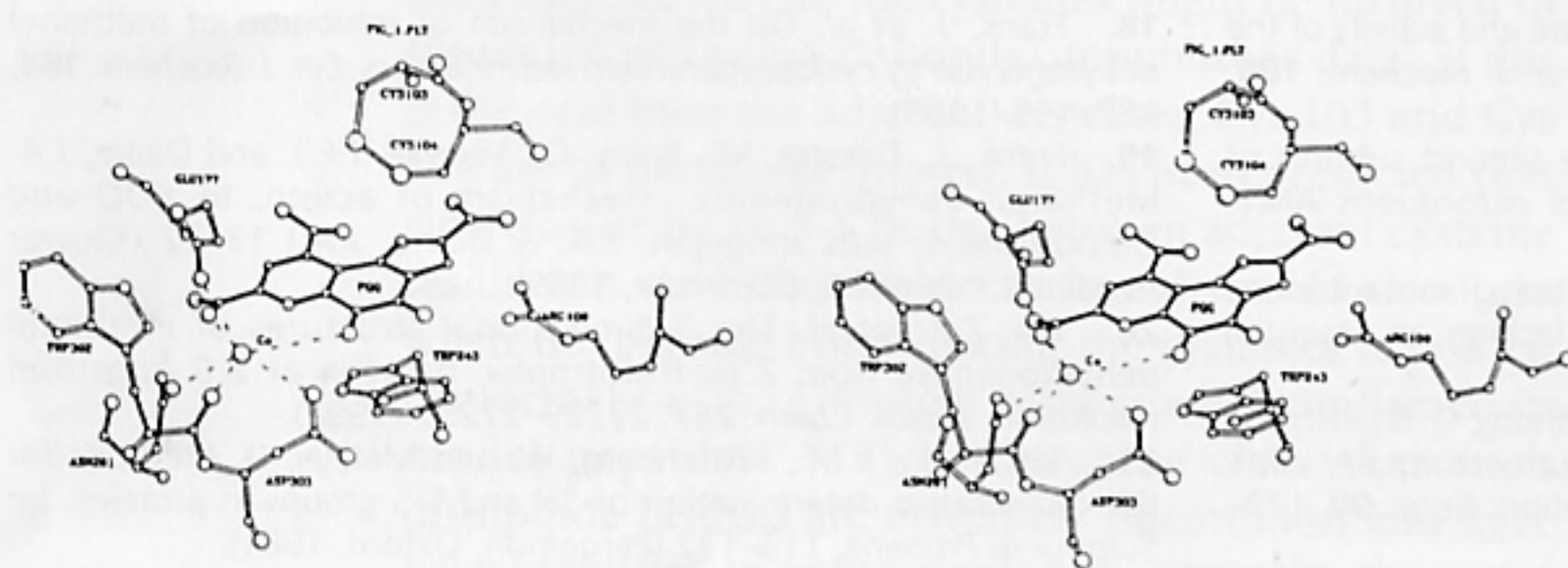


Fig. 3 A stereo diagram of the active site of MDH, showing the relative locations of the PQQ prosthetic group, the Cys 103–Cys 104 disulphide bridge and the calcium ion. The active site is contained entirely within the α -subunit, some distance from both the symmetry related site and the β -chains.

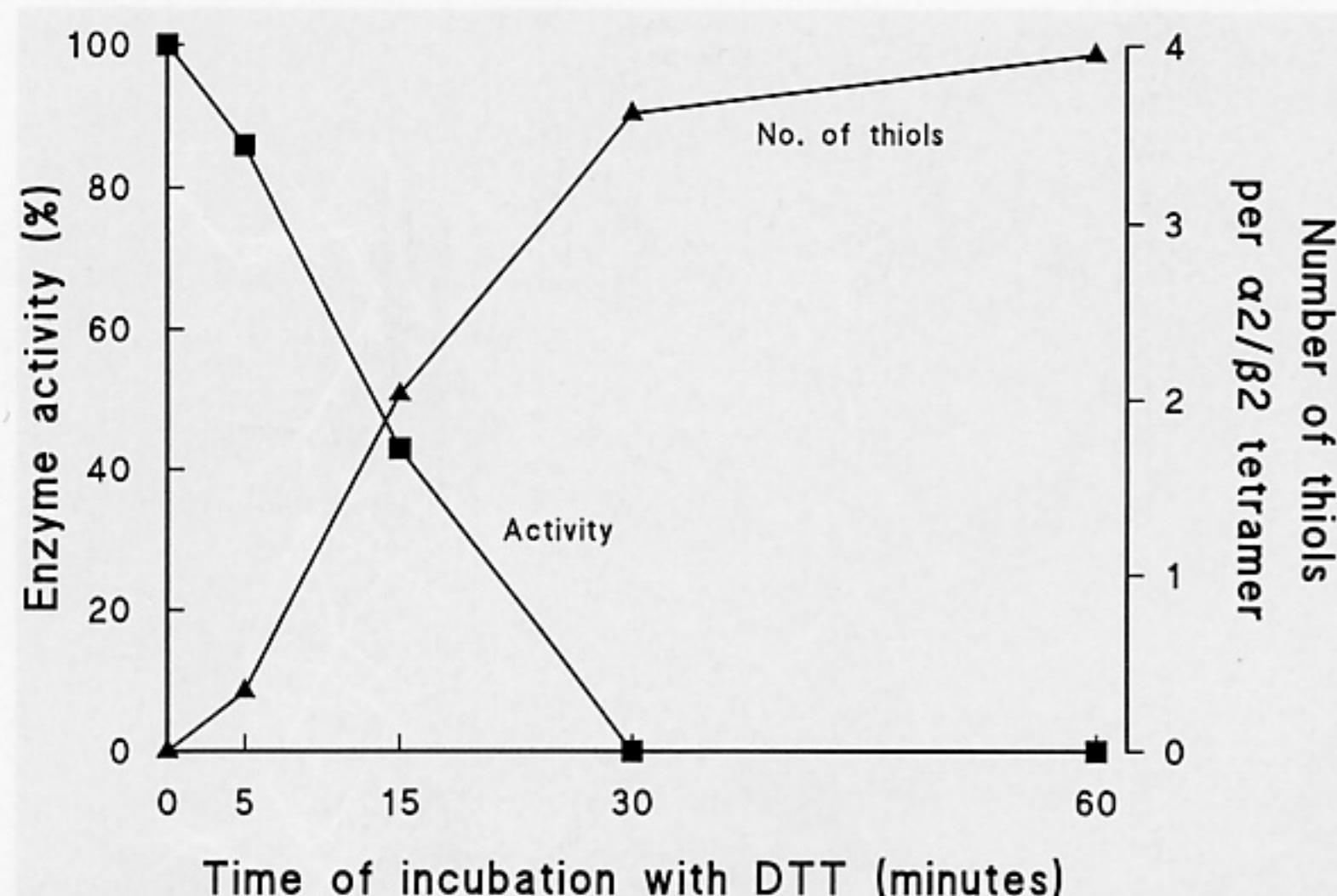


Fig. 4 Plot showing that the reduction of the Cys 103–Cys 104 disulphide bridge (triangles) is correlated with loss of enzyme activity in transferring electrons to cytochrome c_L (squares).

Methods

MDH (2.6 nmol) was reduced with 5 mM DTT in de-gassed Tris buffer (50 mM, pH 8.0) containing 1 mM EDTA, and reagent removed on a Pharmacia G25HR10/10 column. Thiols were determined as described (ref. 21) or by carboxymethylation for 3h with ^3H - or ^{14}C iodoacetate (20 mM). Growth, harvesting and breakage of *M. extorquens* (N.C.I.M.B.9133); purification of methanol dehydrogenase and cytochrome c_L ; and assays of MDH with phenazine ethosulphate or cytochrome c_L were described previously^{7,8,22}. Reduced, carboxymethylated MDH (60 nmol) was digested with trypsin (1/25 ratio w/w) in 50 mM Tris buffer (pH 8) at 37 °C for 16 h. Peptides were separated at 18 °C on a reverse phase C_{18} column using a solvent gradient; solvent A was 0.05% TFA in water, and solvent B was 0.05% TFA in acetonitrile. The radioactive fraction was run on a second reverse phase C_{18} column; solvent A was 10 mM ammonium acetate at pH 7, and solvent B was 90% acetonitrile + 10% solvent A. The bulk of the radioactivity eluted as a single well-separated peak showing no absorbance at 280 nm. The peptide was sequenced using an Applied Biosystem 477A gas phase (pulsed liquid) protein sequencer coupled to a model 120A phenylthiohydantoin-derivative analyser. The electron density map was calculated at 3Å resolution using phases from two isomorphous derivatives, K_2PtCl_4 and ethyl mercuric phosphate (EMP), and symmetry averaging around the non-crystallographic molecular two-fold axis¹⁵. X-ray intensity data were collected on a Marresearch Image Plate mounted with a Ragaku rotating anode X-ray generator. Data were processed using the DENZO programme package. Electron density maps were interpreted using an Evans and Sutherland ESV40 Graphics System, with programmes FRODO and O (refs 23,24), incorporating the known amino acid sequences of the α - and β -chains^{7,9}.

of Trp 302 (Fig. 3), as well as the C7 carboxyl group and the C5 quinone oxygen of PQQ, perhaps allowing the calcium ion to act as a Lewis acid facilitating the nucleophilic attack of substrate at the quinone carbon^{2,16–19}.

The presence of an unusual disulphide bridge, whose reduction leads to loss of activity, and of a Ca^{2+} ion bonded to PQQ in the active site of MDH, raises the question of the individual roles of these features in the activity of MDH, and more particularly how they may be integrated into the catalytic and electron transfer mechanisms.

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